Vitamin E: murine studies versus clinical trials

Domenico Praticò

Center for Experimental Therapeutics and Department of Pharmacology, University of Pennsylvania, Philadelphia, PA, USA

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Vitamin E is the most effective lipid-soluble antioxidant present in mammalian cells. The hypothesis that links vitamin E to atherogenesis postulates that oxidative modifications of unsaturated fatty acids in the low-density lipoprotein particles play a crucial role in the pathogenesis of this chronic disease. Therefore, vitamin E supplementation should reduce the extent of oxidation and, thus, be protective against atherosclerosis.

This hypothesis is strongly supported by studies in murine models of atherosclerosis. In contrast, clinical trials using this vitamin have been giving a more confused picture than expected, with results ranging from a significant protective action to the absence of any effect. However, these findings do not reduce the validity of the "oxidative hypothesis" and of the large body of experimental evidence accumulated so far in its favor. Several differences between animal studies and clinical trials, and among clinical trials themselves are taken into account in order to explain the conflicting findings. Finally, insights into what might be the most appropriate nature of future trials in humans are given.

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Address:

Domenico Praticò, MD

Center for Experimental Therapeutics and Department of Pharmacology University of Pennsylvania BRB II/III, Room 812 421, Curie Boulevard Philadelphia, PA 19104 USA
E-mail: domenico@spirit.gcrc.upenn.edu

Vitamin E is the most important lipid-soluble chain-breaking antioxidant in mammalian cells[1]. It includes two groups of vitamins, tocopherols and tocotrienols, with four structurally related compounds. In humans α-tocopherol predominates and it is considered the most active form of vitamin E. Therefore, the term vitamin E is often identified with α-tocopherol and vice versa. In the last 50 years a large number of studies have conclusively demonstrated that its principal function is to defend tissues against oxidative damage, although other biological properties have been also described[2-4]. The principal dietary sources of vitamin E are vegetable oils, whole grains and nuts[5]. However, no more than 30 IU/day of vitamin E can be obtained from food alone, an amount much lower than the one generally used in interventional studies. Dose-response studies have shown that plasma vitamin E levels in healthy subjects can be increased up to 4 times the basal values[6], and that they are regulated by the α-tocopherol transfer protein, a cytosolic protein highly expressed in the liver[7].

The belief that various chronic diseases may be secondary to oxidative damage[8] explains the renewed and increasing attention paid to this vitamin during the last decade. Among these diseases, atherosclerosis is one of the fields where its biological effects have been extensively investigated *in vitro* and *in vivo*. At the basis of this interest is the "oxidative hypothesis" of atherosclerosis which claims that oxidation of unsaturated fatty acids in the low-density lipoprotein (LDL) particles triggers a series of events that lead to the development of atherosclerotic lesions[9].

In this review article I will present data from animal and human studies on the role of vitamin E in modulating atherogenesis.

Murine studies

As a species, the mouse is highly resistant to atherosclerosis. In an attempt to overcome this problem, this animal has been fed diets containing up to 10-20 times the amount of cholesterol contained in a human diet and an unnatural constituent, cholic acid, which is toxic when fed for a long time to mice. However, through genetic manipulation it has been possible to develop lines of mice that are more susceptible to atherosclerosis. At present, several of these models are available, but two are the most studied: mice lacking the apolipoprotein E (apoE KO) and mice lacking the LDL receptor (LDLR KO). ApoE KO mice fed on chow progressively develop hypercholesterolemia and athero-

sclerotic lesions resembling those observed in humans[10]. This process is exacerbated when mice are fed a high fat diet (0.12% cholesterol and 20% butter fat). In contrast, LDLR KO mice fed on a chow diet do not develop atherosclerosis. However, when they are fed a fat diet, they develop high cholesterol levels and aortic atherosclerotic lesions that range from fatty streaks to advanced atheromas[11]. Surprisingly, the majority of the studies published so far and using these animal models have mostly used combinations of vitamin E and other antioxidant vitamins or different synthetic molecules, such as N,N-diphenylphenylenediamine, with antioxidant properties[12]. However, although it is difficult to compare them, in general they have consistently shown that vitamin E and antioxidants in general are effective in reducing atherosclerosis. In this review I will only present data from studies that have used vitamin E alone or a mixture of antioxidants that included vitamin E.

Crawford et al.[13] showed that a mixture of dietary antioxidants (0.1% vitamin E, 0.5% β-carotene and 0.05% vitamin C) inhibits *ex-vivo* LDL oxidation and fatty streak lesion development in LDLR KO mice. In another study, C57BL/6 mice, naturally resistant to atherosclerosis, were fed a diet containing 0.5% cholic acid supplemented with vitamin E alone (10 IU/g diet) or butylated hydroxytoluene (1% weight), a synthetic antioxidant. Mice fed with vitamin E, despite having a more atherogenic lipid profile, had lesions similar to controls; in contrast, mice fed butylated hydroxytoluene developed a significantly greater area of aortic fatty streaks[14]. The addition of low amounts of synthetic antioxidant vitamins (0.05% α-tocopherol and 0.05% all-trans β-carotene) to the diet had no effect on atherogenesis in apoE KO mice[15]. Remarkably, this result was not associated with any effect on the plasma levels of antibodies against oxidized LDL or on *ex-vivo* LDL oxidation, two indices of lipid peroxidation and oxidative stress which have been correlated with the development of atherosclerosis[9,12]. Taken together, these results suggest that the circulating antioxidant levels reached in these animals with the enriched diet were not sufficient to modulate atherosclerosis *in vivo*. This was secondary to the fact that they were too low to exert an effect on the increased oxidative stress that accompanies the evolution of the disease in this model.

Our group demonstrated that higher levels of vitamin E alone (2 IU/g diet) added to the chow diet of apoE KO mice significantly reduced the aortic atherosclerotic lesion area[16]. Remarkably, this was independent of any effect on plasma lipid levels, but directly correlated with the increase in plasma vitamin E levels and inversely related with the level of circulating and vascular isoprostane $F_{2\alpha}$-VI, a specific marker of *in vivo* lipid peroxidation[17]. Furthermore, we found that in these animals vitamin E significantly reduced the titers of circulating antibodies against oxidized phospholipids[18], another marker of lipid peroxidation that has been correlated with the development of atherosclerosis[19]. These results imply that a threshold level of vitamin E is needed *in vivo* in order to obtain a beneficial effect on atherogenesis which, at least in this animal model, is likely to be mainly secondary to its antioxidant properties.

Consistent with our results, Terasawa et al.[20] reported that apoE KO mice, rendered deficient in vitamin E by deleting the α-tocopherol transfer protein gene, developed aortic atherosclerotic lesions, the severity of which was associated with a significant increase in the F_2-isoprostane levels in aortic tissue. Both studies support the hypothesis that vitamin E is able to modulate atherosclerosis. This effect is mediated by its antioxidant properties, even though other mechanisms might have contributed.

Clinical studies

Compared with the relatively small number of murine studies, the amount of available data on the effects of vitamin E on human atherosclerosis is enormous. They range from epidemiological to prospective observational and clinical interventional studies. One of the first epidemiological studies supporting the antioxidant hypothesis for cardiovascular disease (CVD) is the MONICA study which showed an inverse correlation between plasma vitamin E levels and the risk of ischemic heart disease and stroke, independently of the presence or otherwise of the other classical risk factors[21,22].

Prospective cohort studies have used dietary questionnaires and self-recalled intake of vitamin E. Two large studies of this kind have been performed so far: the Nurses' Health Study and the Health Professional Study. In both, the dietary intake of vitamin E was found to be strongly, but not significantly, correlated with a reduced risk of coronary heart disease or death[23,24]. In contrast, both studies concluded that vitamin C was not protective.

Four controlled interventional trials studying the effect of vitamin E in improving the circulation in the legs of patients with intermittent claudication, one of the most frequent complications of peripheral atherosclerosis, have been performed. Three of them, which enrolled symptomatic patients, found that vitamin E provided significant benefit[25-27]. In contrast, the fourth trial, which used 50 mg of vitamin E for a 4-year follow-up in a cohort of heavy smokers free of symptoms, found that this molecule exerted no effect on the prevention of intermittent claudication[28].

Several large randomized placebo-controlled blinded interventional trials have examined the effects of vitamin E on CVD. However, only three of them had the preventive effect of vitamin E on CVD included among

their primary endpoints; therefore only these will be considered in this review.

The first clinical study, the Cambridge Heart Antioxidant Study (CHAOS), used doses of 400-800 IU/day of vitamin E alone in patients with established and angiographically proven coronary artery disease. The group receiving vitamin E had a significant reduction in the incidence of CVD death and of non-fatal myocardial infarction. This effect was due to a very significant reduction in the risk for non-fatal myocardial infarction. However, there was no significant effect on CVD death alone. This surprising result was later analyzed and it was shown that of the total of 72 deaths, just 6 occurred in the group that was compliant with the vitamin E regimen, whereas 21 occurred in the non-compliant group and 32 in the placebo group[29]. The second study is the Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico (GISSI) Prevention Study, which enrolled patients who survived a myocardial infarction within 3 months of the date of randomization. The primary endpoints were death, non-fatal myocardial infarction and stroke. Subjects received 300 mg of synthetic vitamin E or a mixture of ω-3 polyunsaturated fatty acids or both or neither. Over a 3.5-year follow-up, it was observed that vitamin E had no effect on any of the endpoints[30]. The reasons for the lack of agreement between these two studies are still unclear. However, it is important to point out some differences: 1) the GISSI subjects were presumably on a "Mediterranean" diet rich in antioxidants, whereas the CHAOS ones were on an "English" diet which is poorer in fruits and vegetables; 2) the GISSI subjects were taking CVD-preventive drugs such as statins; therefore a further benefit attributable to vitamin E was probably not to be expected; 3) the CHAOS study used 400-800 IU/day of natural vitamin E, whereas the GISSI study used only 300 mg of synthetic vitamin E, equivalent to 150 mg of natural vitamin E.

Finally, the Heart Outcomes Prevention Evaluation (HOPE) study used 400 IU/day of vitamin E and an angiotensin-converting enzyme inhibitor (ramipril). In this study, the enrolled patients' average age was 65-66 years; they already had some form of lesion developing, and were already diagnosed as being at a high risk for secondary events. The primary endpoints were myocardial infarction, death from CVD and stroke. The study found no significant difference in the number of primary or secondary events between vitamin E-supplemented and placebo groups after a mean follow-up lasting 4.5 years. In contrast, ramipril appeared to be strongly protective[31].

More recently, the results of a primary prevention trial that used 300 mg of synthetic vitamin E (equivalent to 150 mg of natural vitamin E) or low-dose aspirin in subjects with cardiovascular risk factors were published. In this study, while aspirin was protective this low-dose vitamin E regimen was not[32].

Conclusions

It is evident that animal model studies have provided a consistent body of evidence that high-dose vitamin E alone or in combination with other antioxidants is remarkably effective in modulating atherogenesis. This supports the concept that lipid peroxidation is a major contributor to atherosclerosis. However, the usefulness of vitamin E for the prevention of CVD in humans has yet to be definitively proven. This, of course, does not mean that the "oxidative hypothesis" of atherosclerosis is incorrect. Several considerations can be made in order to explain the negative results in clinical trials. The first possibility is that there is a species-related difference between animal and human atherosclerosis such that vitamin E might have a different effect depending on the scenario. Second, it is possible that in mice vitamin E is effective because it is typically administered at an early stage of the disease. In contrast, in humans it has little or no effect because it is prescribed when advanced lesions are already established. Perhaps trials in which the development of new lesions is measured will be more comparable with animal studies. Third, while in most of the animal studies data regarding lipid peroxidation and/or oxidant status before and after vitamin E intake are available, the same may not be said of clinical trials. It is possible that in some patients the rate of lipid peroxidation is higher than in others, and that they will hence be more responsive to the treatment. Therefore, before administering antioxidants, the oxidant status of the patients to be enrolled should be checked. These are some of the issues that need to be addressed in future studies.

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